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RNA helicase Ddx5 and the noncoding RNA SRA act as coactivators in the Notch signaling pathway

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ABSTRACT

Notch signaling plays a pivotal role in embryonic and postnatal development.
Upon binding of a Notch ligand, proteolytic cleavage events liberate the Notch-intracellular domain (NICD) that migrates into the nucleus. In order to activate target genes, NICD associates with the transcription factor RBP-J (also known as CSL), Mastermind and the acetyltransferase p300.
Here, we identify the DEAD-box RNA helicase Ddx5 as a novel component of the RBP-J/NICD complex utilizing a biotinylation-tagging approach followed by mass-spectrometry. Biochemical assays confirm a direct interaction of Ddx5 with RBP-J. We show that Ddx5 localizes at RBP-J binding sites within the Notch target genes preTCRα, Hes1 and CD25 in a Notch-dependent manner. Moreover, knockdown of Ddx5 also downregulates a subset of Notch target genes in a murine pre T-cell model. Interestingly, also knockdown/overexpression of the RNA coactivator SRA, a cofactor of Ddx5, downregulates Hes1 and preTCRα. Using Chromatin-IP, we show that this effect is accompanied with a loss of p300 occupancy at Notch target genes and decreased histone acetylation. Together, our data demonstrate that Ddx5 and SRA function as coactivators of Notch signaling.

1. Introduction

The Notch signaling pathway is an evolutionarily conserved pathway that plays pivotal roles in many cellular and developmental processes. Notch signaling is activated upon cell-to-cell contact as a result of interactions between Notch receptors and their ligands (Delta or Jagged). This promotes two proteolytic cleavage events of the Notch receptor [1]. The extracellular cleavage is catalyzed by ADAM-metalloproteases, followed by the intracellular cleavage mediated by a γ-secretase containing complex. The second cleavage releases the Notch intracellular domain (NICD), which translocates into the nucleus to activate the transcription of its targets [2]. The NICD cannot directly bind to DNA but heterodimerizes with the DNA binding protein RBP-J (recombination signal binding protein Jκ also called CSL or CBF1) [3] activating the transcription of genes containing RBP-J binding sites. The interaction of NICD with RBP-J creates an interface that is recognized by the essential coactivator MAML [4] reviewed in [5]. The RBP-J/Notch/Mastermind coactivator complex can subsequently recruit the histone acetyltransferase p300 or PCAF [6,7].

Biochemical studies by Capobianco and colleagues showed that the Notch-coactivator is a high molecular weight complex of 1.5 MDa in size, suggesting that there are additional, yet unknown coactivator components [8]. The importance of MAML has been shown with dominant-negative MAML, which completely blocks Notch-mediated transcriptional activation [9]. In addition, several kinases and the ubiquitin ligase Fbw7 destabilize this complex, which is important not only during development, but also during carcinogenesis [10].

Ddx5/p68 is an active DEAD-box containing RNA helicase that has been proposed to play a tumor-promoting role and has been described as a transcriptional coactivator and a splicing factor, reviewed in [11,12]. Ddx5 expression appears to be ubiquitous but expression levels vary considerably between tissues. A closely related RNA helicase, Ddx17, forms heterodimers with Ddx5. Ddx5 has been described as an important coactivator of estrogen-receptor alpha where it interacts with a long non-coding RNA called steroid receptor RNA activator (SRA) [13,14]. Interestingly, Ddx5 together with SRA also interact with CCCTC-binding factor (CTCF) and cohesins [15].

In this study, we show that Ddx5 is found in complex with the RBP-J/Notch coactivator complex and is required for transcriptional

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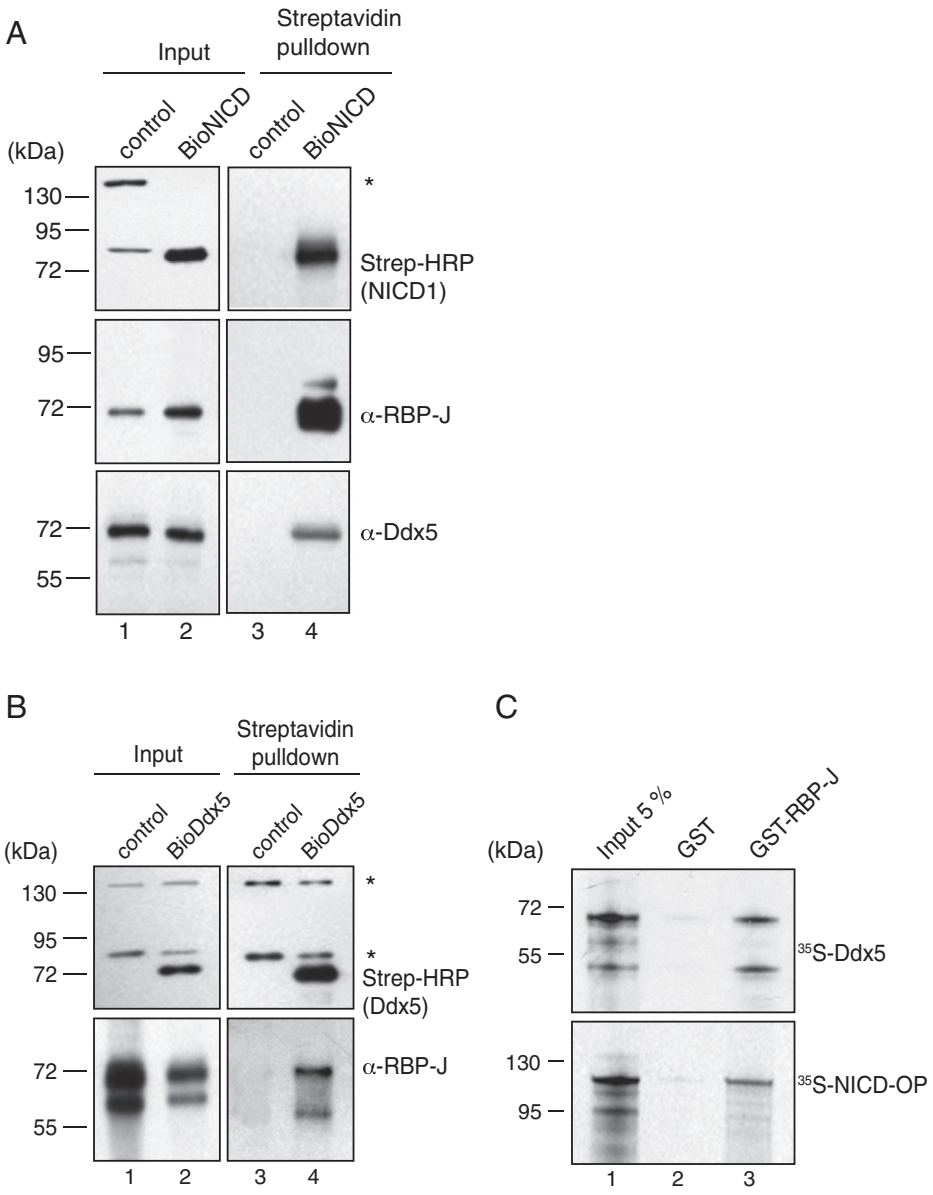


Fig. 1. Ddx5 physically interacts with the RBP-J/Notch complex in vivo and in vitro. A. Notch and Ddx5 interact in coimmunoprecipitation experiments: biotin-tagged Notch intracellular domain (NICD) was expressed in preT-cells called Beko. Streptavidin magnetic beads were used to pulldown Notch-ICD coactivator complex. Precipitated proteins were analyzed by Western blot using streptavidin-HRP detecting Notch-ICD (upper panel), α-RBP-J (middle-panel) and α-Ddx5 antibodies (lower panel). In lane 4 Ddx5 co-purifies with biotin-NICD. B. Ddx5 and RBP-J interact in coimmunoprecipitation experiments: Beko cells expressing biotin-tagged Ddx5 were subjected to streptavidin pulldown. Ddx5 was detected by Western blotting with Strep-HRP (upper panel) and endogenous RBP-J with α-RBP-J antibodies (lower panel, lane 4). As control cells for A and B, Beko cells expressing the biotin ligase (BirA)-only were used and the in vivo biotinylated background proteins (carboxylases) are marked with asterisks. C. RBP-J and Ddx5 interact in GST pull-down experiments: Cell-free synthesized ^{35}S-labeled Ddx5 binds to GST-RBP-J immobilized to glutathione-Sepharose beads (upper panel, lane 3), but not to GST only. As positive control the interaction of RBP-J with NICD-OP (Notch intracellular domain with OPA and PEST (OP) domain) was examined (lower panel, lane 3).

activation of several Notch target genes. In addition, we reveal that the non-coding RNA SRA plays a similar role and is required for the recruitment of histone acetyltransferase p300 to Notch target genes.

2. Material and methods

2.1. Reagents, antibodies and cell culture

For ChIP experiments, the following antibodies were used: H3K18ac (Cell Signaling, 9675), H3 (Abcam, ab1791), p300 (Santa Cruz Biotechnologies, sc-585), Ddx5 (Diagenode, PAb 204). For Western blotting, the following antibodies were used: RBP-J (Cosmo Bio Co., Clone T6709; secondary:α-rat IgG [Dianova]), Ddx5 (Diagenode, PAb 204), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Abcam, ab8245), RNA pol II (N-20) (Santa Cruz Biotechnology, sc-899), α-Flag (M5 monoclonal, Sigma, F4042), secondary: anti-mouse IgG (Amersham Biosciences, NA931), α-rabbit IgG (Cell Signaling, 7074).

Mouse pre-T cells (Beko) were grown in Iscove's modified Dulbecco medium (Gibco) with 2% fetal calf serum FCS (Pan-Biotech), 1% nonessential amino acids (Gibco), 0.3 mg/L Primatone (Quest), 1% penicillin/streptomycin (Gibco) and 5 mg/L insulin (Sigma). The cell line was maintained at 37 °C under 5% CO_2. The cells were treated with GSI (10 μg/mL DAPT; Calbiochem (565770) or DMSO only. For tamoxifen induction, the cells were treated with 0.75 μg/mL 4-OHT (Sigma, H7904) or 100% ethanol as control [16]. HeLa cells (ATCC CCL 2) were grown in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented

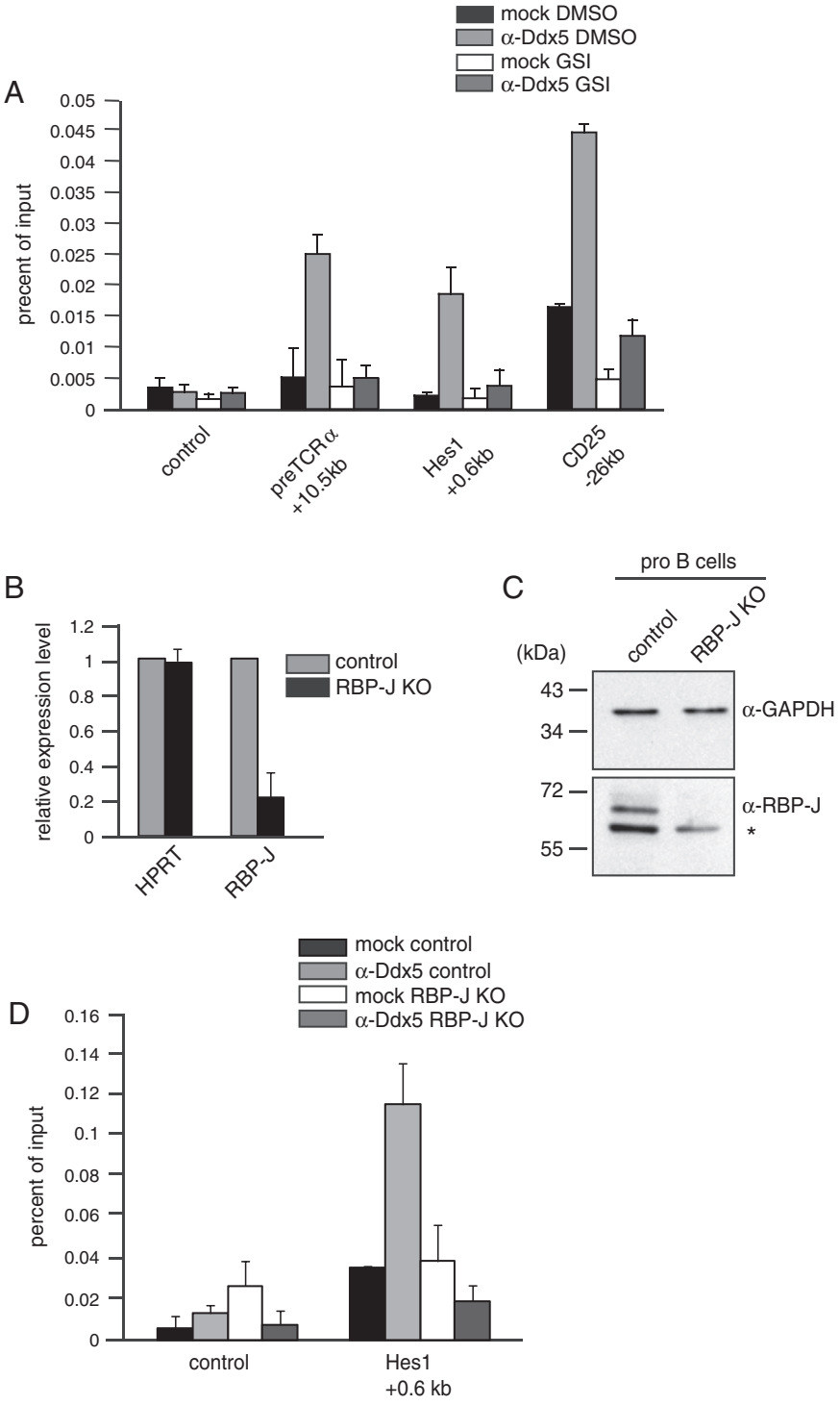


Fig. 2. Ddx5 localizes at enhancer elements of Notch target genes in a Notch- and RBP-J dependent manner. A. Chromatin-immunoprecipitation (ChIP) with α-Ddx5 antibody. Only in the active state (DMSO control) is Ddx5 localized at enhancer elements of Notch target genes preTCRα (+10.5 kb), Hes1 (+0.6 kb) and CD25 (−26 kb) (light gray bars). Data were normalized to GAPDH promoter region, for which no occupancy was observed; the Hes1 region +2.4 kb served as an additional control. Upon inhibition of Notch by treatment with γ-secretase inhibitor DAPT for 24 h, Ddx5 occupancy is undetectable (dark gray bars) (mean ± SD, $n = 3$). B. RBP-J transcript is downregulated in RBP-J deficient proB-cells. The knockout (KO) of RBP-J is induced upon tamoxifen treatment. Upon tamoxifen treatment of RBP-$J^{flox/flox}$ x Cre-ER proB-cells, the mRNA level of RBP-J is severely diminished (black bars) compared with control (ethanol treated cells, gray bars) and housekeeping gene HPRT. The data are normalized versus the housekeeping gene TBP. Data presented as means ± SD of three independent experiments measured in duplicates. C. RBP-J protein is lost in RBP-J deficient proB-cells. Using RBP-J specific antibody in Western blot the RBP-J protein disappears in RBP-J knock out cells (lower panel) compared with *GAPDH* control (upper panel). Background bands are labeled with asterisks. D. Ddx5 binding at Hes1 Notch target gene is RBP-J dependent. ChIP experiments in RBP-J knock out (KO) proB-cells were performed using α-Ddx5 antibody. When RBP-J is deleted, Ddx5 no longer binds to the Hes1 enhancer region +0.6 kb. ChIP was normalized to GAPDH promoter region and as control the Hes1 region +2.4 kb was analyzed. Values are presented as mean ± SD for duplicate samples from a representative experiment; the experiment was repeated three times.

with 10% fetal calf serum (FCS), penicillin and streptomycin. The cell line was maintained at 37 °C under 5% CO_2. Pro-B cells from RBP-$J^{flox/flox}$ ×Cre-ER mice were established by culturing bone marrow cells for 14 days in IL-7–supplemented Iscove's medium (Biochrom) containing 10% FCS (Pan-Biotech), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen) and 5×10^{-5} M 2-mercaptoethanol. The cells were maintained at 37 °C under 7.5% CO_2. For IL-7–dependent growth, the supernatant of J558L cells stably transfected with a vector encoding mouse IL-7 was supplemented in excess.

Mouse Ddx5 was amplified using Beko preT cell cDNA and the following primers (forw: GCGGCCGCTATATCGAGTTATTCTAGTGACCGA GACCGCGGCC, rev: GCGGCCGCTTATTGAGAATACCCTGTTGGCATGG GATAG). The resulting fragment was cut with NotI and ligated into the retroviral vector pMyIRESGFP with biotin tag. The following human Ddx5 fragments were kindly provided by Dr. Ralf Janknecht (University of Oklahoma Health Sciences Center): pcDNA3-p68, pcDNA3-p68K144N, pGEX 2T p68 (2–80), pGEX 2T p68 (80–178), pGEX 2T p68 (176–282), pGEX 2T p68 (282–388), pGEX 2T p68 (387–500), pGEX 2T p68 (500–614) [17]. The expression plasmids for NICD [pcDNA3-mNotch1-IC + OP (1751–2531)] and RBP-VP16 (pSG5-RBP-VP16) were described previously [6]. Mouse SRA was amplified using Beko preT cell cDNA and the following primers (forw: CCTCGAGCAATTCTCCTACGGGCTCCA, rev: AGACTCACTTCACCTGACTT CCTGCGGCCGCC. The resulting fragment was cut with XhoI, NotI and ligated into the retroviral vector pLxsp-puro. The pSCT-SRAhuman and pSCT-empty were kindly provided by Dr. Charles

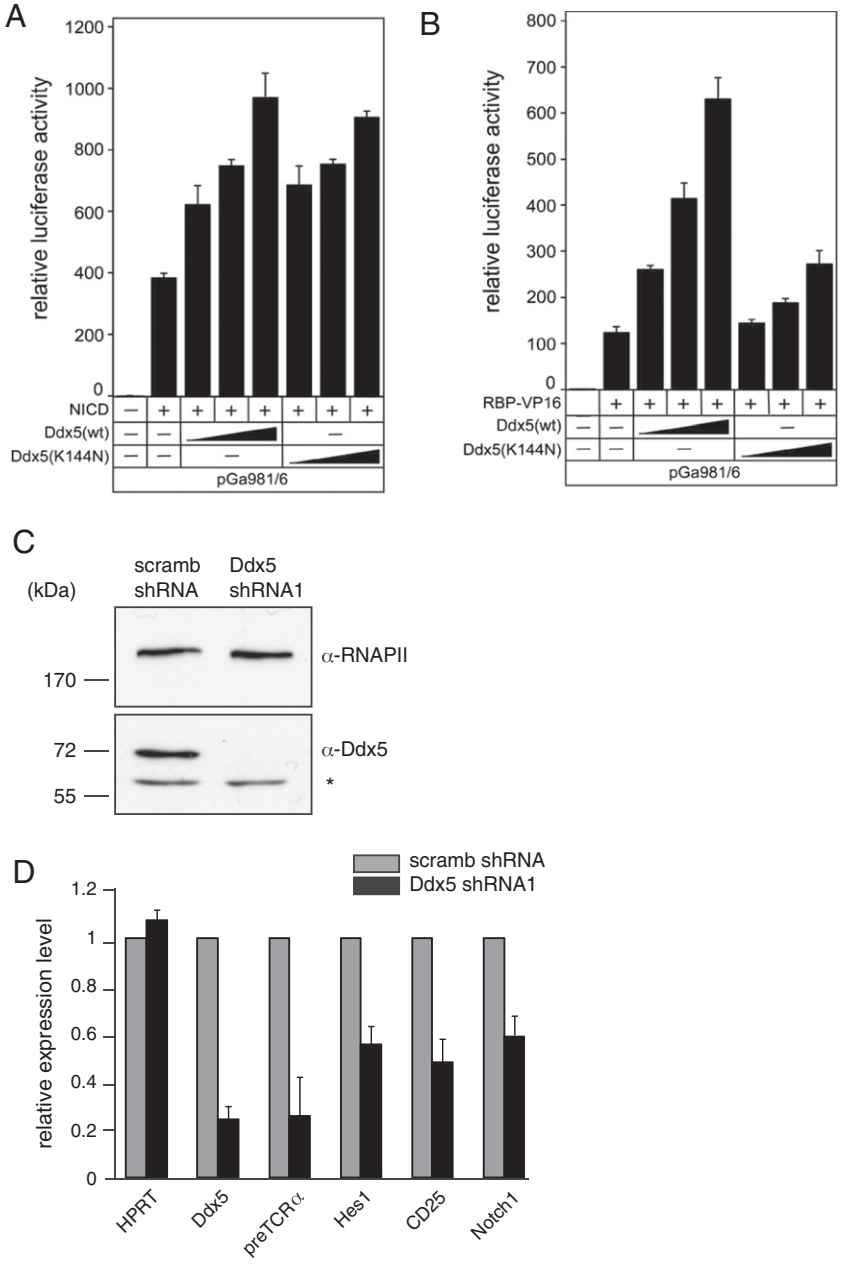
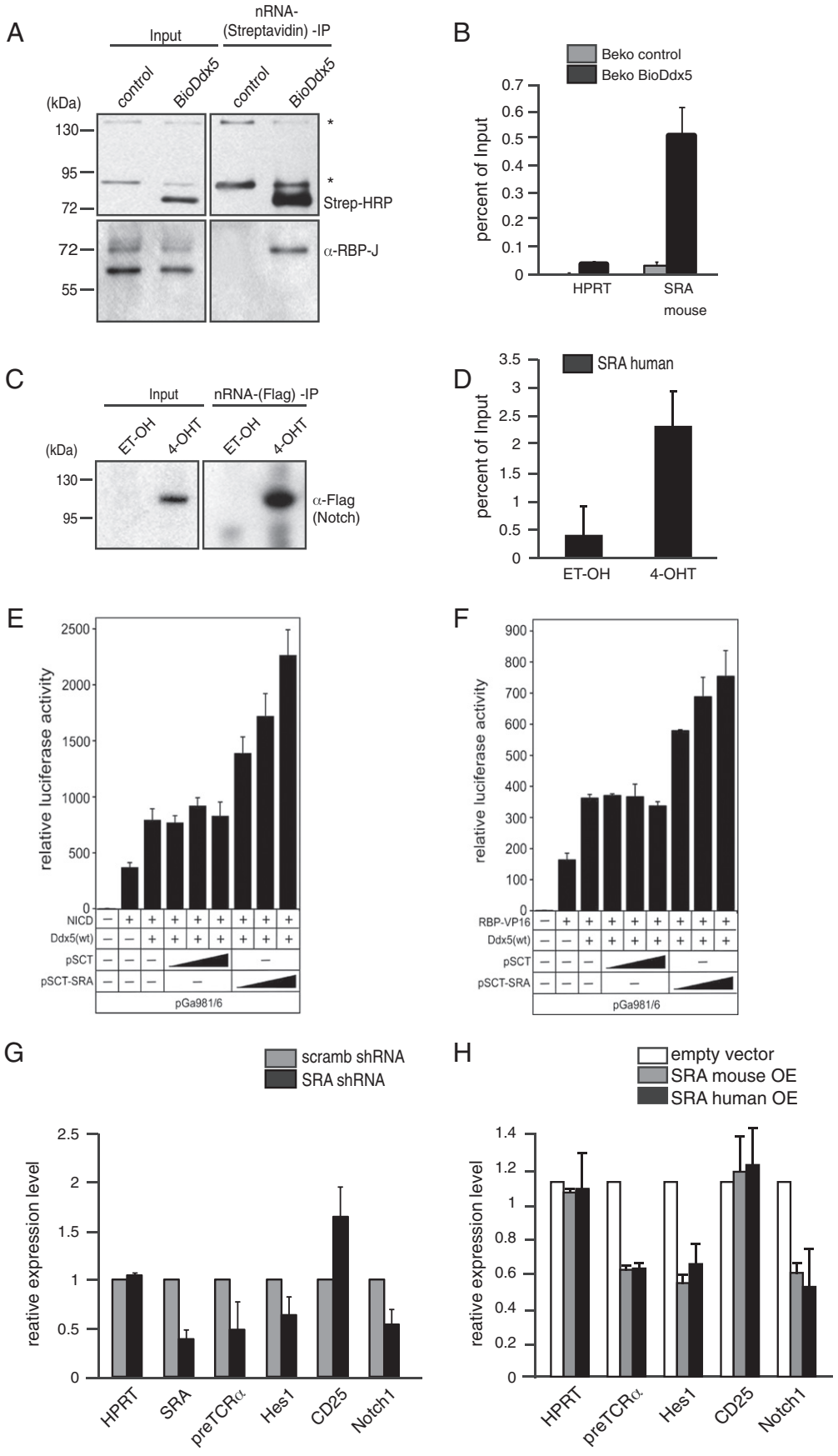


Fig. 3. Ddx5 acts as a coactivator in Notch signaling. A and B. Ddx5 acts as a coactivator in Notch/RBP-J dependent transcription. A. Notch responsive reporter construct [pGa981/6, (1 μg)] was transfected alone or together with expression plasmids (25 ng) for the murine Notch intracellular domain [(Notch-ICD] (A), RBP-VP16 (B) and increasing amounts of expression plasmid for Ddx5(wt) (100 ng, 200 ng, 300 ng) or helicase dead Ddx5(K144N) into HeLa cells. Promoter activity was analyzed 24 hours after transfection (mean ± SD, $n = 4$). C. Western blots revealed a significant downregulation of Ddx5 protein in shRNA transfected preT cells Beko (lower panel). RNAPII antibody served as a loading control (upper panel). Unknown bands are labeled with an asterisk. D. Knockdown of Ddx5 leads to downregulation of several Notch target genes. RT-PCR revealed a significant downregulation of Ddx5 in preT cells Beko transfected with Ddx5 specific shRNA1 (black bars) as compared with control scrambled shRNA (gray bars). The expression of Notch target genes *preTCRα*, *Hes1*, *CD25* and *Notch1* is decreased upon Ddx5 knockdown (mean ± SD, $n = 3$).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Hes1
CD25
Notch1

Foulds (Baylor College of Medicine, Houston) [13]. Furthermore the pSCT-SRAhuman was used as a template for amplification with following primers (forw: CCTCGAGGGCAACAAGGAACGCGGCT, rev: TTTCAATAAAAACATCTCTGCGGGCGGCCGCC). The resulting fragment was cut with XhoI, NotI and ligated into the retroviral vector pLxsp-puro. The retroviral pMIGRFlagNotchER plasmid, the GST-RBP-J and the pcDNA3.1-RBP-J fragments (NTD, BTD, CTD) were described previously [16].

2.2. Retroviral infection of Beko cells

Ecotropic-Phoenix cells were transfected with calcium phosphate method. Therefore Phoenix cells were pre-treated with 25 μM chloroquine for 5 min. For transfection 1 ml 2× HBS (pH 7.05), 124 μl 2 M $CaCl_2$, 20 μg DNA up to 1 ml with ddH2O were transferred onto the cells, mixed thoroughly and incubated over night. The next day fresh medium was loaded onto the cells. After 24 h incubation Beko cells were retrovirally infected using supernatants from Ecotropic-Phoenix packaging cells, supplemented with polybrene at 2 mg/ml. Retroviral gene expression was monitored using flow cytometry to measure co-expressed fluorescent proteins. Where necessary, cells were sorted to obtain pure populations with equivalent expression levels.

2.3. Knockdown in Beko cells

For knockdown of Ddx5 and SRA the Lentiviral Harvard shRNA (TRC1) library was used with the following target sequences in the vector backbone pLKO.1-puro: Ddx5 shRNA1 (CGGGAAGCTAATCAAGCAATT), Ddx5 shRNA2 (GCGAATGTCATGGATGTGATT), SRA (CAGACTCACTTCACCTGA CTT) and scrambled shRNA was used as control. For transfection, 293 T cells were incubated 6 h together with 30 μl Lipofectamine-2000 reagent (Invitrogen), 3.3 μg DNA (pLKO.1-puro) and with the lentiviral packaging vectors psPAX (2.5 μg) and pMD (1 μg). After 48 h incubation the supernatant from 293T cells was added to the Beko cells for the lentiviral infection. The puromycin selection was carried out 24 h after infection by adding 2 μg/ml puromycin to the cells.

2.4. RNA extraction, RT–PCR and quantitative PCR

Total RNA was isolated from Beko cells using Trizol reagents (Ambion, 15596018). For cDNA synthesis, 1 μg of RNAs was reverse transcribed using random hexamers and reverse transcriptase according to the manufacturer's protocol (Thermo Scientific, K1622). Real-time PCR reactions were performed using a 7300 ABI PRISM sequence detector system (Applied Biosystems) according to the manufacturer's recommendations. Quantitative PCRs were performed using ABsolute QPCR ROX mix (Thermo Scientific, no. AB-1139), gene-specific oligonucleotides (see Supplementary material) and double-dye probes under the following conditions: 2 min at 50 °C and 15 min at 95 °C, and then 45 cycles of 15 sec at 95 °C and 1 min at 60 °C.

For RNA immunoprecipitation experiments quantitative PCRs were performed using ABsolute QPCR SYBR Green ROX mix (Thermo Scientific, no. AB-1163) under the following conditions: 2 min at 50 °C and 15 min at 95 °C, and then 50 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 1 min 70 °C followed by dissociation. The results were normalized to endogenous TBP (TATA-binding protein) mRNA expression level.

2.5. ChIP

Ddx5, p300 and H3K18ac ChIP experiments were performed via cross-linking ChIP using the protocol provided by Upstate Biotechnology with minor modifications. Briefly, Beko cells were fixed with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped with 125 mM glycine. Chromatin was prepared by using a cell lysis buffer (1% SDS, 50 mM Tris–HCl at pH 8.0, 10 mM EDTA, 2 mm dithiothreitol) and sonicated. Chromatin was 5-fold diluted with dilution buffer (16.7 mM Tris–HCl at pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton X-100) and precleared with presaturated Protein A beads. The precleared chromatin extract was incubated overnight with the appropriate antibody and 50 μl were collected as input control. The immunoprecipitates were immobilized on protein A-Sepharose beads for 1 h. After washing the beads with 150 mM NaCl, 500 mM NaCl and 0.25 M LiCl-containing buffers, chromatin was eluted from the beads with elution buffer (0.1 M $NaHCO_3$, 1% SDS) at 25 °C shaking. The cross-linking of chromatin was reversed for 6 h at 65 °C in the presence of 200 mM NaCl. Chromatin was dissolved in Tris–EDTA buffer (40 mM Tris–HCl at pH 6.5, 20 mM EDTA), and treated with proteinase K for 1 h at 45 °C. After purification by phenol-chloroform extraction, the chromatin was precipitated overnight at −20 °C in the presence of tRNA, glycogen and isopropanol. The enriched chromatin was analyzed by quantitative real time PCR. The values were calculated as percent of the input.

2.6. Preparation of nuclear cell extracts

Ten million cells were washed three times in PBS and pelleted by centrifugation at 300 *g*. The pellet was resuspended in 1 ml of ice-cold hypotonic buffer (20 mM Hepes, 20 mM NaCl, 5 mM $MgCl_2$, 0.2 mM PMSF) incubated 20 min on ice and vortexed 20 s afterwards. After 5 min centrifugation at 20,000 *g* the pellet was washed once in PBS and resuspended in 100 μl hypertonic buffer (0.2% NP-40, 20 mM Hepes, 300 mM KCl, 1 mM $MgCl_2$ 1 mM Dithiothreitol (DTT), 1× Proteaseinhibitor (Roche) and 0.2 mM phenylmethylsulfonyl-fluoride (PMSF). After 20 min incubation the lysate was cleared at 80,000 *g* for 30 min. Protein concentrations were determined by the Bradford assay (Bio-Rad) and extracts were used for immunoprecipitation, RNA-immunoprecipitation and for Western blotting.

2.7. Streptavidin-immunoprecipitation

Immunoprecipitation experiments were carried out using nuclear extract from Beko preT cells. 500 μg extracts were incubated with 10 μl streptavidine magnetic beads (Dynabeads M-280, Invitrogen

Fig. 4. The non-coding RNA SRA has distinct effects in Notch signaling. A and B: SRA associates with Ddx5 in native RNA-immunoprecipitation (nRNA-IP) experiments. A. Nuclear extracts from Beko preT cells stably transfected with biotin-tagged Ddx5 were used in streptavidin coimmunoprecipitation experiments and either analyzed by immunoblotting with streptavidin-HRP (biotin tagged-Ddx5, upper panel) and anti-RBP-J antibody (lower panel), or B, total RNA was extracted and subjected to qRT-PCR with the primers specific for SRA and HPRT (mean ± SD, $n = 3$). As control cells, preTcell line Beko expressing biotin ligase (BirA) were used and the *in vivo* biotinylated background proteins (carboxylases) are marked with an asterisk. C and D: SRA associates with Notch in native RNA-immunoprecipitation (nRNA-IP) experiments. C. Beko preT cells stably transfected with Flag-tagged NICD-ER and human SRA were treated 24 h with tamoxifen (4-OHT) or ethanol (ET-OH) as control. Nuclear extracts were prepared and used in Flag-immunoprecipitation experiments and analyzed by immunoblotting with anti-Flag antibody (Flag tagged-Notch) or D, total RNA was extracted and subjected to qRT-PCR with the primers specific for human SRA (mean ± SD, $n = 3$). Data was normalized to control HPRT. E and F: The long noncoding RNA SRA facilitates Ddx5/Notch/RBP-J dependent transcription. A Notch responsive reporter construct [pGa981/6, (1 μg)] was transfected alone or together with expression plasmids (25 ng) for the murine Notch intracellular domain [(NICD, (E)], RBP-VP16 (F) and expression plasmid for Ddx5(wt) (200 ng) and increasing amounts of empty pSCT vector or pSCT-SRA (100 ng, 200 ng, 300 ng) into HeLa cells. Promoter activity was analyzed 24 hours after transfection. MW, SD from at least four independent experiments are shown (mean ± SD, $n = 4$). G. Knockdown of the long non coding RNA SRA leads to slight downregulation of several Notch target genes. qRT-PCR analysis of transcripts in SRA-depleted (black bars) Beko preT cells compared with control scrambled shRNA (light gray bars). The Notch target genes *preTCRα*, *Hes1* and *Notch1* are slightly downregulated whereas *CD25* is upregulated. Data was normalized to TBP (mean ± SD, $n = 3$). H. Overexpression of SRA leads to downregulation of several Notch target genes. Total RNA was extracted from Beko preT cells stably transfected with SRA mouse (light gray bars) or SRA human (black bars) plasmids. The Notch target genes *preTCRα*, *Hes1* and *Notch1* were downregulated whereas *CD25* remained unchanged compared with empty vector control (white bars). Data was normalized to TBP (mean ± SD, $n = 3$).

112.06) for 2 h at 4 °C. The beads were washed six times with streptavidin washing buffer (TBS 1×, 250 mM NaCl, 0.2% NP-40) and were resuspended in SDS-polyacrylamide gel loading buffer.

2.8. Western blotting

The proteins resolved in SDS-polyacrylamide gels (6–12%) were transferred electrophoretically for 1 h at 4 °C temperature to nitrocellulose membranes (Whatman) at 380 mA by using a Tris–glycine buffer system. The membranes were preblocked for 1 h in a solution of 5% milk powder in PBS before the antibodies were added.

2.9. GST pull-down assay

Proteins were translated in vitro in the presence of ^{35}S methionine using the reticulocyte lysate-coupled transcription/translation system (Promega). Translation and labeling quality were monitored by SDS-PAGE. The GST fusion proteins were expressed in *Escherichia coli* strain BL21 (Stratagene) and stored as whole bacterial lysates at −80 °C. Approximately, 3 μg of GST protein and GST fusion protein were immobilized with glutathione–Sepharose beads (GE Healthcare, no. 17-5132-01) and incubated together with the in vitro translated proteins in buffer A (40 mM Hepes at pH 7.5, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Nonidet P40 [NP-40], 100 mM KCl) under rotation for 1 h at 4 °C. Beads incubated with in vitro translated Ddx5 were washed two times with 600 μl of buffer B (equivalent to buffer A, but containing 300 mM KCl) and two times with 600 μl of buffer C (equivalent to buffer A, but containing 500 mM KCl). Beads incubated with in vitro translated NICD-OP (Notch intracellular domain with OPA and PEST (OP) domain) were washed two times with 600 μl of buffer A and two times with 600 μl of buffer B [18]. As control 100 U Benzonase (Novagen, 70746) and 100 U RNase (Stratagene, 400720) were added to the 1 h binding reaction.

2.10. Native RNA-immunoprecipitation (nRNA-IP)

Beko preT cells were transfected with biotin-tagged Ddx5 and nuclear extract was prepared. For purification 5 mg extract was incubated 2 h with streptavidin magnetic beads. The beads were washed 4 times with RIP washing buffer (50 mM Tris–HCl pH 7.5, 750 mM NaCl, 1 mM $MgCl_2$, 0.05% NP-40) and afterwards resuspended in 100 μl RIP washing buffer. 10 μl was used for western blot analysis and resuspended in 10 μl SDS-polyacrylamide gel loading buffer. The other 90 μl were incubated for 30 min 55 °C with 1% SDS and 30 μg proteinase K. The RNA purification was carried out using the Qiagen RNeasy Kit (Cat. no. 74134) and cDNA synthesis was performed with the Thermo Scientific Kit (RevertAid M-MuLV Reverse Transcriptase K1629). Beko preT cells transfected with FlagNotchER and human SRA were treated 24 h with 4-OHT and nuclear extract was prepared. For purification 5 mg extract was incubated 2 h with α- Flag M2 Agarose beads (Sigma A2220) and washed 6 times with RIP washing buffer. The following procedure was done like above.

2.11. Luciferase assay

HeLa cells (5×10^4) were transfected in 24-well plates (Nunc) with 1 μg of reporter plasmid pGa981/6 [19,20] alone or together with various amounts of expression plasmid (given in the corresponding figure legends). Luciferase activity was determined from four independent experiments 24 h after transfection with an LB 9501 luminometer (Berthold) using the luciferase assay system from Promega.

3. Results

3.1. RNA helicase Ddx5 physically interacts with the RBP-J/NICD complex

In order to identify Notch-intracellular domain (NICD) coactivator components, we isolated NICD-containing complexes taking a biotinylation tagging approach [21]. Using extracts from pre T-cells infected with bio-NICD, Notch-containing complexes were purified in a single-step procedure using streptavidin magnetic beads followed by mass-spectrometric analysis. Notch1, Notch2, Mastermind-1, Mastermind-3 and Dtx-2 peptides as well as a novel coactivator component, RNA helicase Ddx5, were present in pulldowns with streptavidin beads. Interestingly, Ddx5 has been previously identified to form a complex with p300 acetyltransferase [22] a known component of the Notch coactivator complex [6]. Mass-spectrometric results were validated by co-immunoprecipitation experiments (Fig. 1A) and GST-pulldown experiments (Fig. 1C and Fig. S1). After streptavidin pulldown using nuclear extracts from bio-NICD expressing cells, Notch-1 was detected in Western blotting using a streptavidin-HRP (Fig. 1A, upper panel, lane 4). In addition to NICD, endogenous RBP-J (middle panel) and Ddx5 (lower panel) were detected using α-RBP-J and α-Ddx5 antibodies. In the reverse pulldown experiment using nuclear extracts from bio-Ddx5 expressing pre-T cells, we could detect endogenous RBP-J (Fig. 1B, lower panel, lane 4). We further tested the physical interaction between Ddx5 and RBP-J in GST pulldown experiments. In vitro transcribed full-length Ddx5 readily interacts with bacterially expressed GST-RBP-J but not with GST only (Fig. 1C). We mapped the RBP-J/Ddx5 interaction extensively on both sides using further GST-pulldown experiments (Fig. S1). Fragments of Ddx5 fused to GST (GST-Ddx5 2–80, 282–388 and 387–500) interact with in vitro transcribed/translated RBP-J (Fig. S1A). When using fragments of RBP-J, the NTD- and BTD-domains but not the CTD domain interact with Ddx5 (Fig. S1B and C). Furthermore, we could show that additional RNase/DNase treatment has no effect on the interaction of Ddx5 and GST-RBP-J (Fig. S2A and B). Together, our data support the notion that Ddx5 is a novel component of the RBP-J/NICD coactivator complex.

3.2. RNA helicase Ddx5 is found at Notch target genes in vivo

To study Ddx5 recruitment at Notch target genes, we used a spontaneous T-cell lymphoma line from TCR-β knockout (Beko) mice as our experimental model system [16]. We compared Beko cells untreated (Notch-on) and Beko cells treated with γ-secretase inhibitor (GSI) for 24 h (Notch-off). In ChIP-experiments we detected strong binding of Ddx5 to RBP-J/NICD binding sites of the genes preTCRα- (+10.5 kb), Hes1 (+0.6 kb) and CD25 (−26 kb) (Fig. 2A). The RBP-J/Notch binding sites are based on ChIP data followed by deep sequencing [23]. Importantly, Ddx5 binds in a Notch-dependent manner, since no binding is observed upon switching-off Notch signaling with GSI (Fig. 2A). In order to verify whether or not Ddx5 recruitment depends on RBP-J, we established a pro-B cell line from RBP-J conditional knockout mice [24] that carry Cre-recombinase transgene fused to the ligand-binding domain of the estrogen receptor, Cre-ER (Fig. 2B–D). By adding tamoxifen, Cre-ER translocates to the nucleus, deleting the exons of RBP-J encoding for the DNA binding domain. RNA and protein expression of RBP-J is lost upon Cre-induction by tamoxifen [25] (Fig. 2B and C). Although Hes1 expression was unaffected in RBP-J knockout B-cells (data not shown), we could observe a strong decrease in Ddx5 binding at the RBP-J enhancer site of Hes1 (+0.6 kb) (Fig. 2D). Ddx5 binding was not observed at +2.4 kb in the Hes1 or at the GAPDH promoter (Fig. 2D).

3.3. RNA helicase Ddx5 acts as a co-activator in Notch signaling

To analyze the function of Ddx5 in Notch/RBP-J dependent transcription we first performed reporter gene assays using the Notch-

dependent pGa981/6 luciferase construct. As shown previously [6,26] co-transfection of both, NICD (Fig. 3A) as well as RBP-VP16 (Fig. 3B) resulted in a clear induction of promoter activity. Increasing amounts of Ddx5(wt) led to a further increase of luciferase activity. Interestingly, the helicase dead Ddx5 mutant [Ddx5(K144N)] also further increased the pre-activated luciferase activity, although to a lesser amount in RBP-VP16 dependent transcription (Fig. 3B). This is in agreement with data analyzing the role of Ddx5 in ERα-Runx2- and p53-mediated transcriptional transactivation [14,27,28]. In order to investigate the role of Ddx5 at endogenous Notch target genes, we generated Ddx5 knockdown cells (Fig. 3C and D, S3A and B). Ddx5 protein levels were significantly downregulated as demonstrated in Western blotting using α-Ddx5 antibodies whereas control antibodies, RNA polymerase II showed no changes (Fig. 3C); similarly Ddx5 mRNA is downregulated (Fig. 3D). Notch target genes preTCRα, Notch1, Hes1 and CD25 are downregulated in Ddx5 knockdown cells, further supporting that Ddx5 functions as a coactivator for Notch (Fig. 3D and Fig. S3B).

3.4. The long non-coding RNA SRA associates with Ddx5 and Notch-ICD in T-cells and is a positive regulator of Notch signaling

Previous studies have shown that Ddx5 interacts with the long non-coding RNA SRA [14]. Using bio-Ddx5 expressing cells, we could observe that not only RBP-J is precipitated (Fig. 4A) but also the long non-coding RNA SRA (Fig. 4B) as demonstrated by native RNA immunoprecipitation. Importantly, in RNA immunoprecipitation experiments using FlagNotchER we observed a tamoxifen-dependent complex formation of Notch and SRA (Fig. 4C and D). This suggests that Ddx5 and SRA are part of the RBP-J/NICD complex.

Using reporter gene assays in HeLa cells we observed an SRA specific activation of Ddx5/NICD/RBP-J dependent transcription (Fig. 4E and F). Coexpression of Notch-ICD (Fig. 4E) or RBP-VP16 (Fig. 4F) together with a modest amount of Ddx5 (200 ng) revealed a slight increase of reporter gene activity (compare with Fig. 3A and B). Transcriptional activation was further enhanced by increasing amounts of SRA expression, but not by the empty vector (pSCT). Importantly, the expression of endogenous SRA is not affected upon knockdown of Ddx5 (Fig. S3C).

Subsequently, we investigated the role of non-coding RNA SRA in promoting activation of Notch target genes. Therefore, we knocked-down SRA in Beko cells and observed a decrease in expression of preTCRα, Notch1 and Hes1 (Fig. 4G). Surprisingly, overexpression of either human or mouse non-coding RNA SRA also leads to a downregulation of preTCRα, Notch1 and Hes1 (Fig. 4H). One possible scenario is a squelching mechanism, in which overexpressed SRA titrates away an important cofactor for Notch. The negative squelching effect was initially described for Gal4-mediated transcriptional activation in yeast [29]. Overexpression of one transcriptional activator is able to sequester a limiting factor leading to repression rather than activation. Subsequently, similar effects were described in higher eukaryotes, i.e. for cFos-Jun [30] and p63 [31].

3.5. Occupancy of p300 and histone acetylation is lost upon SRA knockdown and overexpression at Notch target genes

We investigated the possible underlying mechanism of transcriptional repression by overexpression and knockdown of SRA. Histone acetyltransferase p300 has been described as an enhancer mark [32] and as a co-activator for Notch-mediated transcription [6]. In ChIP-experiments using SRA knockdown Beko cells, p300 recruitment is

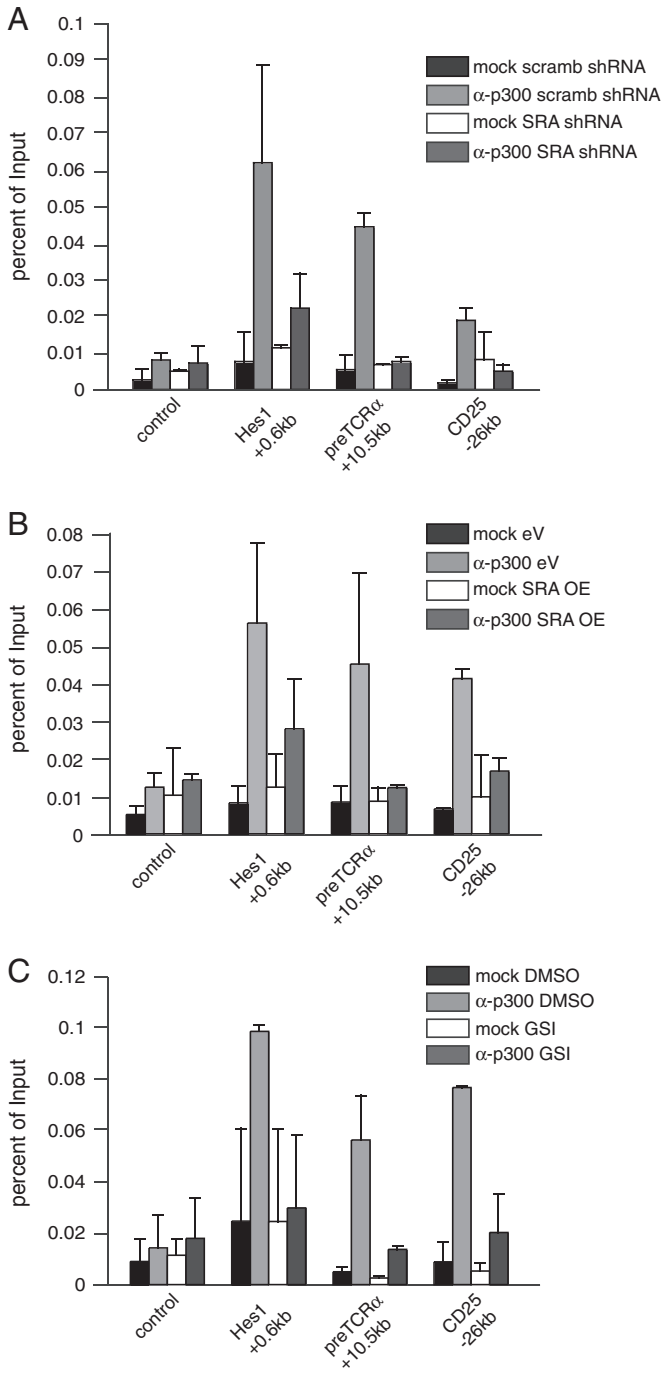

Fig. 5. Recruitment of histoneacetyltransferase p300 is lost upon either knockdown or overexpression of SRA. ChIP analysis was performed with an α-p300 antibody at enhancer elements of Notch target genes. A. Pre-T-cell line Beko was infected with shRNA SRA in order to downregulate SRA expression. Chromatin-immunoprecipitation (ChIP) experiments using anti-p300 antibodies (no antibody as mock control) were performed in Beko cells treated with SRA specific shRNA or scrambled shRNA. p300 was significantly enriched around the enhancer elements of Notch target genes preTCRα+10.5 kb, Hes1+0.6 kb and CD25-26 kb (light gray bars) and lost upon SRA knockdown (dark gray bars). ChIP was normalized to the GAPDH promoter and no occupancy was found at Hes1 region +2.4 kb (control). B. Pre-T-cell line Beko was infected with a plasmid for SRA overexpression. ChIP experiments using anti-p300 antibodies (no antibody as mock control) were performed in the absence (empty vector, eV) or after SRA overexpression (SRA plasmid). p300 was significantly enriched around the enhancer elements of Notch target genes preTCRα+10.5 kb, Hes1+0.6 kb and CD25-26 kb (light gray bars) and erased upon SRA overexpression (OE) (dark gray bars). ChIP was normalized to the GAPDH promoter and no occupancy was found at Hes1 region +2.4 kb (control). Data presented as means±SD of three independent experiments. C. In ChIP experiments using α-p300 antibodies, the recruitment of p300 to enhancer elements of Notch target genes preTCRα+10.5 kb, Hes1+0.6 kb and CD25-26 kb is abrogated upon 24 hour GSI treatment (dark gray bars). No antibody was used as mock control. ChIP was normalized to the GAPDH promoter and no occupancy was found at Hes1 region +2.4 kb (control). Data presented as means±SD of three independent experiments measured in duplicates.

severely reduced at Notch target genes Hes1 and preTCRα and to a lesser extent at CD25 (Fig. 5A). Interestingly, loss of p300 recruitment is also reduced after overexpression of SRA (Fig. 5B) which is in line with the downregulation of Notch target genes (see Fig. 4G and H). The loss of binding of p300 at Notch target genes is comparable to what we observe after Notch pathway inhibition by GSI (Fig. 5C). Surprisingly, this is not the case in Ddx5 knockdown cells (Fig. S4); see also discussion.

Histone acetylation at lysine 18 of histone H3 (H3K18), a known mark for p300 acetylation [33], is also reduced upon either overexpression or knockdown of SRA (Fig. S5A and B). Thus, noncoding RNA SRA mediates p300 recruitment and H3K18 acetylation at Notch target genes.

4. Discussion

Activation of the Notch pathway is implicated in several disease states, including acute T-cell lymphoblastic leukemia [10] and chronic lymphocytic leukemia [34]. Thus, finding new targets for the Notch coactivator complex is desirable to develop Notch inhibitors or modifiers. In this regard enzymes in particular are of great interest. Our identification of RNA helicase Ddx5 as coactivator of Notch target genes suggests that inhibitors of Ddx5 could be used pharmacologically to target Notch-mediated diseases. In a recent publication by Dr. L. Wu and colleagues, Ddx5 has been shown to directly interact with MAML1, a well-known cofactor for Notch [35]. These results are in agreement with our ChIP results showing that Ddx5 recruitment is Notch-dependent. The data presented here shows that Ddx5 also directly interacts with the transcription factor RBP-J, suggesting that there are at least two docking sites in the Notch-coactivator complex for Ddx5. We clearly find Ddx5 to interact with RBP-J in the absence of Notch activation, i.e. pulling down RBP-J followed by mass spectrometry (data not shown). In in vivo ChIP experiments Ddx5 is found at target genes exclusively in a Notch-dependent manner. This might be due to other coactivator components like Mastermind. However, in Notch inactive B-cells we do observe RBP-J dependence of Ddx5 occupancy at Notch target genes. Together, this suggests that there is also a Notch-independent mechanism of RBP-J/Ddx5 interaction.

It will be highly interesting to further investigate the role of non-coding RNAs in the activation of Notch target genes. We show that the long non-coding RNA SRA plays a key role in the regulation of several Notch target genes. Moreover, it would be of interest to follow the dynamic recruitment of SRA at Notch target genes; however, techniques from such experiments have yet to be developed.

In the case of knockdown or overexpression of the coactivator SRA the effects on Notch target genes are relatively broad, and p300 recruitment at Notch target genes is severely affected. In the case of Ddx5 knockdown only a subset of Notch target genes (Hes1, preTCRα and Notch1) are downregulated. Expression of other Notch target genes like Dtx1, NRARP and Hey1 remain unchanged. Surprisingly, recruitment of the acetyltransferase p300 remains unaffected at downregulated genes. Since Ddx5 not only regulates p300 recruitment and histone acetylation, but also post-transcriptional events such as splicing, nuclear export or stabilization of RNA [11], one possible scenario is that the homologous RNA helicase Ddx17 can partially compensate for lack of Ddx5, but importantly, not for SRA. This is supported by a study from Janknecht and colleagues showing that Ddx17 interacts with p300 [17]. Thus, the mechanism for how specificity at Notch target genes is achieved remains an open and demanding question.

Recently, Fuller-Pace and colleagues published a study about Ddx5/p68 conditional knockout mice [36]. Here, a strong effect on p53-dependent p21 expression and cell-cycle arrest after DNA damage is observed. It will be interesting to see the effect of Ddx5-deletion in hematopoiesis, especially during early T-cell development.

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